COMMENTARY

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Phagosome proteomics

A powerful tool to assess bacteria-mediated immunomodulation

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Bacteria-mediated immunomodulation has important implications in microbial infection and bacterial vaccines. Intraphagosomal bacteria negotiate survival niches with the intracellular environment by modulating the phagosome composition during invasion. The final phagosome composition determines the fate of the intraphagosomal bacterium or the efficacy of a bacterial vaccine. Therefore, the phagosome proteome is a valuable readout to assess the ability of a natural or genetically engineered bacterial strain to modulate the host immune response. Compared to a preparation of latex-bead-containing phagosomes, the preparation of bacterial phagosomes requires additional measures to ensure comparable purity due to their closer density to some other organelles. This bottleneck can be overcome with delicate preparation protocols, proper experimental designs to facilitate bioinformatics based discrimination against contaminating proteins, and the incorporation of stable-isotope labeled internal standards to correct for contaminating fractions of phagosomal proteins. The rapid progress in the proteomics and bioinformatics fields provides an array of techniques that promise to bring about an unprecedented coverage of both known and as yet undiscovered immunomodulation pathways within bacterial phagosome proteomes. A precise portrait of the bacteria-mediated immunomodulation pathways in phagosomes will likely aid in the intelligent design of bioengineered bacterial vaccine strains for important future biomedical applications.

Key words: phagosome, proteomics, immunomodulation, bacterial pathogen, mass spectrometry, intracellular bacteria, host-pathogen interaction, lysosome, antigen presentation, antigen processing

Abbreviations: BCG, bacillus calmette-guérin; Mtb, *Mycobacterium tuberculosis*; FASP, filter assisted sample preparation

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Intraphagosomal Bacterial Pathogens

Bacterial infections remain a major cause of human diseases. A bacterial infection involves a series of biological activities from both bacterial and eukaryotic organisms. Causative bacteria interact with host cells either extracellularly or intracellularly. While extracellular bacteria evade phagocytosis and often secrete toxins to cause diseases, intracellular bacteria invade host cells and replicate intracellularly to cause active infection.

Some intracellular bacteria, such as *Mycobacterium tuberculosis* (Mtb), are highly successful in using the intraphagosomal environment as a sanctuary to evade the host immune defense for persistent infection. Interestingly, it is also well established that certain bacteria, especially facultative anaerobes, prefer tumor to normal tissue for replication.[1] The bacterial preference of tumor tissues for replication suggests that host functions of immune surveillance and clearance are suppressed in the tumor environment, providing a sanctuary for those bacteria. Thus, bacterial infections might in turn modulate host immune response to work against cancerous cells.[2] The ability of certain bacteria to act against cancerous cells probably results from the long history of host-pathogen interaction.[3] The realized benefit of bacteria-mediated immunomodulation to fight off cancers can be traced back to the discoveries made by Dr. William Coley a century ago.

A host defense against intraphagosomal bacterial infection involves a phagocytosis process that typically sends bacteria to

lysosomes by a programmed series of membrane fusion events occurring between the bacterial vacuole and host endocytic vesicles. Successful intraphagosomal bacteria have acquired genetic determinants that allow them to exploit host niches as bacterial environments. Those intraphagosomal pathogens have developed strategies to avoid delivery to lysosomes. The strategies include disrupting endocytic maturation and subverting other vesicular transport pathways in the host cell.[4] Different bacterial pathogens might use different evasion strategies to avoid the host's antimicrobial actions to thrive inside a host cell.[5]

The ability of some intraphagosomal bacterial pathogens to avoid being delivered to lysosomes and to suppress antigen processing and presentation pathways poses an obstacle for vaccine development. For example, although Bacillus Calmette-Guérin (BCG) has remained the only approved vaccine against tuberculosis disease since its first use in humans in the early nineteen twenties, the vaccine is known to have large variability in efficacy among different populations (0–80%) and to be ineffective in protecting adults from tuberculosis.[6] BCG is capable of resisting the phagosome-lysosome fusion and of downregulating the host antigen processing and presentation pathways. Such a capability of BCG to suppress the host antigen processing and presentation pathways contributes to its reduced efficiency. An induction of autophagy with rapamycin enhances the BCG vaccine efficacy, confirming the importance of delivering BCG to a degradative compartment for better vaccine efficacy.[7] A genetically engineered recombinant BCG strain designed for cathepsin S secretion also restores the intracellular proteolytic activity to stimulate the expression of antigen processing and presentation pathway molecules.[8]

Phagosomes are Self-Sufficient Antigen Processing Organelles

Phagosomes are fully competent antigen-processing organelles.[9] They are capable of processing intracellular bacterial antigens for presentation via both MHC class I and II pathways. MHC class I molecules could present exogenous antigens to elicit an MHC class-I-dependent $CD8^+$ T-cell response by cross-presentation.[10] Proteomic analysis of phagosomes showed that all proteins needed for all steps in cross-presentation are assembled on the phagosome to enable cross-presentation.[11] A recent work by Boulais et al. demonstrates that a proteomic comparison of latex-bead-containing phagosomes from three distinct organisms reveals the evolution of phagosomal protein networks that enabled the transformation of the phagosome from a phagotrophic compartment into an organelle fully competent for antigen presentation.[12]

Antigen processing in phagosomes is generally thought to require acidification of phagosomes to activate proteolytic enzymes like cathapsins. The acidification of phagosomes occurs when phagosomes fuse with late endosomes and subsequently with lysosomes to lead to the formation of the acidified compartment called the phagolysosomes. Vacuolar-type H^+-ATPases play a central role in this acidification process.[13] Because of the importance of phagosome acidification or maturation in antigen processing, a live attenuated Mtb strain that allows limited phagosome maturation renders it a more effective vaccine candidate than BCG.[14] The limited phagosome maturation inhibits active replication of a vaccine candidate within the phagosome. Meanwhile, the limitation of phagosome maturation delays the destruction of the intraphagosomal bacterium, allows the synthesis of unique intraphagosomal bacterial antigens, and allows the infected host cell to migrate to other sites to elicit proper protective immunity.

The encounter between host antigen presentation cells and some highly successful intraphagosomal bacteria is not well understood. For example, although Mtb possesses multiple mechanisms to inhibit the action of interferon gamma[15] and to prevent phagosome maturation, it still elicits a vigorous immune response upon invading macrophages. This is typical of invasive bacteria acting on defined cells of the immune system that are often the primary targets of microbial assault.[16] Therefore, it is highly possible that host cells in the immune system possess alternative mechanisms to process and present antigens from intraphagosomal pathogens. Phagosomes are the critical location where such alternative antigen processing pathways function to recognize intraphagosomal pathogens of varied virulence.

The abundance of antigen processing and presentation enzymes assembled on the phagosome most likely correlates with the virulence of a bacterial pathogen. Weakening an intraphagosomal bacterium by genetic engineering or an antibiotic treatment might result in further phagosome maturation,[14] which works in synergism to lead to either better vaccine efficacy or eventual eradication of the intraphagosomal bacterium. Therefore, the phagosome proteome serves as a valuable readout to evaluate vaccine candidates and drug treatments against intraphagosomal bacteria.

Bacterial Phagosome Proteomics: The Bottleneck and the Promise

Proteomic studies on phagosomes have contributed significantly to our understanding of phagosome biogenesis and immunity-related functions.[17] Proteomic analysis of phagosomes showed that proteins needed for antigen processing and presentation, including those involved in cross-presentation, are assembled on phagosomes.[11] Proteomics also shows the potential to discover novel components involved in phagosome functions including antigen processing and presentation.[18] Jutras et al. found that γ-secretase is a functional phagosome component that resides on newly formed phagosomes and remains associated with them throughout their maturation into phagolysosomes.[19] With proteomics, Nicastrin was found to be present on BCG- and latex-bead-containing phagosomes in human THP-1 macrophages[20] as well as on phagosomes from Mtb infected mouse bone-marrow-derived macrophages.[21] Nicastrin is a member of the γ-secretase that is an integral membrane-bound protease complex.[19] While Notch and the amyloid precursor protein are well-known substrates of γ-secretase, other γ-secretase substrates remain to be uncovered. The finding that Nicastrin was enriched on Mtb phagosomes provides an interesting lead to its functional role in phagosomes.[21]

Despite great potential, proteomic analysis of bacterial phagosomes is impeded by the significant bottleneck of obtaining sufficient amounts of material with a high purity.[22] Bacteria-containing phagosomes typically require purification with multiple passes of differential density centrifugation because they have densities closer to some other organelles. On the other hand, latex-bead-containing phagosomes can be obtained with a higher purity in a single-step differential density centrifugation.

The general approaches used today to purify bacteria-containing phagosomes evolved from those described earlier, e.g., by Sturgill-Koszycki et al. Additional measures have also been employed to reduce the contamination from other organelles. In a study of Legionella-containing phagosomes, Shevchuk et al. eliminated lysosomes with colloidal iron-loading and magnetic separation, eliminated mitochondria by iodophenylnitrophenyltetrazolium heavy labeling, and degraded nucleic acid with Benzonase.[24] In addition to differential density gradients, magnetic separation was also used to purify phagosomes containing magnetically labeled mycobacteria.[25]

Electron microscopy has been used to examine the structure-level details of a phagosome preparation that may reveal entrapment of contaminating membrane fragments or organelles in phagosome preparations.[26] With a higher degree of sensitivity in LC/MS-based proteomic analysis of phagosomes,[11] however, trace contaminants might also be detected. Therefore, a significant level of detail will be required to pinpoint which phagosome proteins contain contaminating fractions from other organelles. Lee et al. used radio-isotope labeled whole cell lysates that never saw latex bead or bacterial particles as an internal standard to estimate the degree of contamination from other organelles during a phagosome preparation.[20] The approach estimated the number of contaminating protein spots on a 2D gel but did not provide a quantitation of the contaminating fraction of individual proteins. However, it can be adapted for using stable-isotope labeling instead of radioactive labeling of host cells to estimate the contaminating fraction of individual proteins in a phagosome preparation. A stable-isotope labeled whole lysate of host cells that never see bacteria or bead particles can be used as an internal standard to mix with unlabeled whole cell lysates that contain bacterial phagosomes to purify. The purified bacterial phagosomes are analyzed with LC/MS to quantify the labeled and unlabeled fractions of a protein.[27] The proteins that only have the unlabeled fraction are not contaminated while those with a labeled fraction can be estimated for the degree of contamination. The contaminating fraction can be corrected as necessary. This approach to quantify unlabeled and labeled fractions of a phagosomal protein was used to determine the turnover of proteins in latex-bead-containing phagosomes.[27]

In addition to the above mentioned biochemical measures to improve purity of a bacterial phagosome preparation, a proper experimental design can also help to increase the confidence in the purity of a detected phagosome protein. For a time course study, the organelle preparations of early and late phagosomes are identical thus contaminants should not fluctuate over time.[27,28] For a comparison of multiple phagosome preparations, proteins from contaminating organelles should not differ among different phagosome preparations that are made under the same conditions.[21] With such experimental designs to focus on differentially regulated proteins, the proteins found differentially abundant among different phagosome preparations or at different time points are more likely to be authentically associated with phagosomes rather than from contaminating organelles.[22]

Advances in the proteomics field have offered multiple options for phagosome proteome analysis with extensive coverage. It has been widely proved that different proteomic approaches often identify different protein subpopulations. Thus, a combination of different fractionation options (**Fig. 1**) will maximize the coverage of a phagosome proteome as it would do for any other proteomes in general. It is conceivable that two typical routes can be taken to analyze phagosome protein samples with LC/MS (**Fig. 1**). The first route will be to digest each unfractionated phagosome protein sample with a proteolytic enzyme such as trypsin to generate a peptide mixture based on the filter-assisted-sample-preparation (FASP) protocol suitable for a membrane protein analysis.[29] For a fast survey of samples, the tryptic digest can be directly injected for LC/MS analysis (option a).[18] For an in-depth analysis, the tryptic peptide mixture is either fractionated offline followed by LC/MS analysis of each fraction,[29] or injected into a MudPIT (multidimensional protein identification technology) system[30] that has been widely used to perform integrated two-dimensional liquid chromatography separation and LC/MS analysis (option b). In the second route, a phagosome protein mixture is first separated based on protein isoelectric focal points (option c) or molecular weights (option d). Each protein fraction is then digested with trypsin to yield peptides for subsequent LC/MS analysis.

It remains an open question how many proteins can eventually be extracted and characterized from a phagosome.[22] A recent in-depth proteomic analysis of latex-bead phagosomes identified 2,415 phagosome proteins with a high level of sensitivity.[11] However, many proteins in some key immunity-related pathways still remain undetected. For example, only 31 were detected from the 91 proteins predicted to be involved in antigen processing and presentation pathways in the KEGG Pathway database (www.genome.jp/kegg/). Thus, the number of proteins from a phagosome preparation will probably be more than that detected by Trost et al.[11] Wisniewski et al. showed that over 1,000 membrane proteins could be identified in a single LC/MS run without sample prefractionation and an extended coverage of a hippocampus proteome was achieved with 4,206 proteins identified from duplicate LC/MS runs of six peptide fractions.[29] We also showed that about 1,000 proteins could be quantified from mycobacterial phagosomes without sample fractionation prior to LC/MS runs.[18] Therefore, the rapid progress in proteomics and bioinformatics promises to deliver comprehensive and quantitative coverage of a phagosome proteome to serve the purpose of assessing bacteria-mediated immunomodulation at the organelle level.[21]

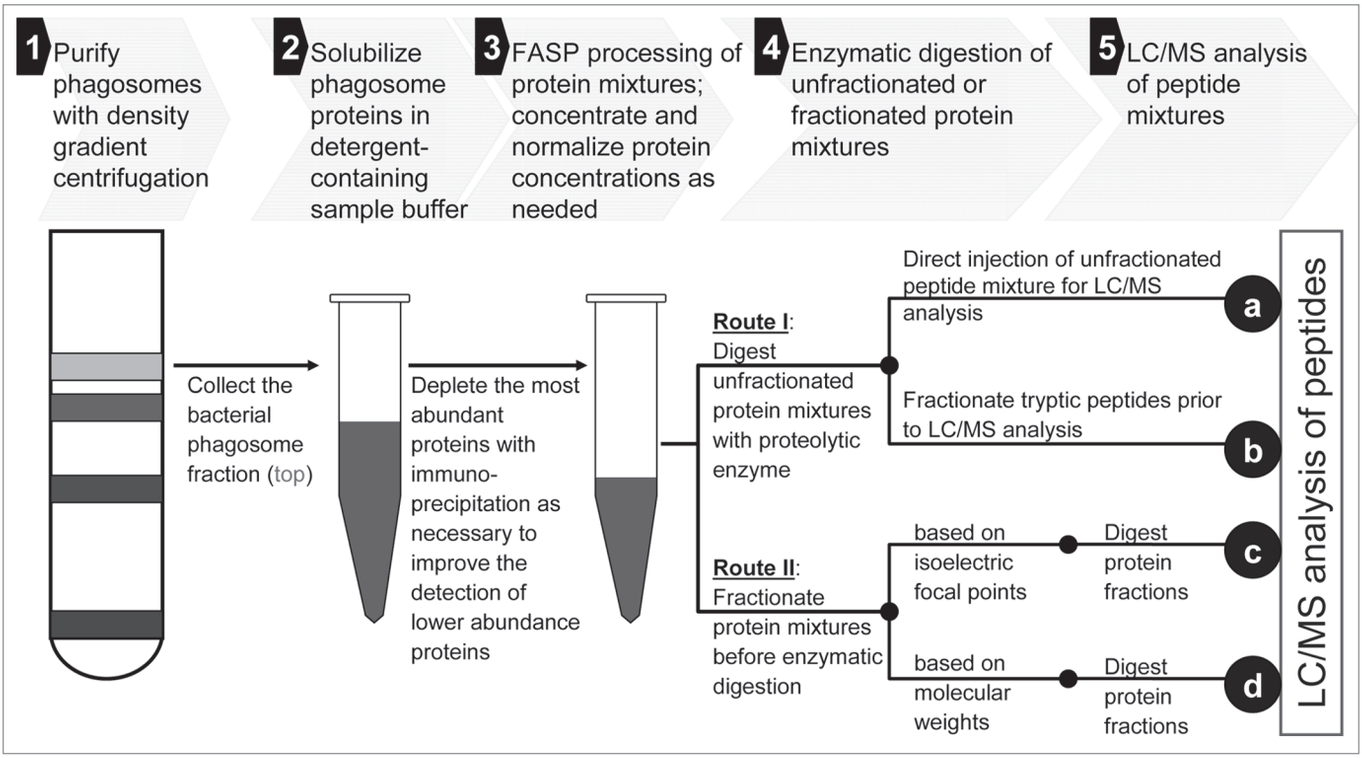

Figure 1. Multiple approaches to process phagosome protein samples for LC/MS-based proteomic analyses. Shown at the top are the five major steps starting from bacterial phagosome preparation to LC/MS analysis of digested peptide mixtures. At step 4, two routes can be taken to generate peptides for LC/MS analysis. In route I, unfractionated proteins are digested into peptide mixtures that are either directly injected for LC/MS analysis (option A) or fractionated prior to LC/MS analysis (option B). In route II, protein mixtures are fractionated based on either isoelectric focusing points (option C) or molecular weights (option D) prior to enzymatic digestion into peptides for subsequent LC/MS analysis.

Conclusion

Intraphagosomal bacteria-mediated immunomodulation has important implications in microbial infection and bacterial vaccines. While a virulent bacterium such as Mtb can significantly inhibit phagosome maturation to suppress an antigen processing process, it is also capable of co-opting the host immune response to form granulomas as a sanctuary for persistent infection. In addition, the typical requirement of live bacteria for vaccination also suggests the importance for a proper level of bacterial immunomodulation to take effect before an effective antigen presentation can occur at the right place and the right time. A systems approach is required for a better understanding of such a sophisticated interplay between a bacterium and the host immune defense.

Phagosome compositions are a direct indicator to decipher the intraphagosomal bacteria-host cell interaction. But, due to certain technical difficulties and technological limitations, a comprehensive survey of bacterial phagosome constituents and pathways has rarely been done at a large scale to facilitate a systematic evaluation of bacterial immunomodulation at the organelle level.

Phagosome proteomics is a powerful approach to study the host-pathogen interaction. Although pure bacterial phagosomes are typically more difficult to prepare than latex bead phagosomes, this bottleneck can be overcome by several means including delicate preparation protocols, proper experimental designs to facilitate bioinformatics-based discrimination against contaminating proteins, and incorporation of stable-isotope labeled internal standards to correct contaminating fractions of phagosomal proteins. The sensitivity and accuracy of modern proteomics and bioinformatics technologies have now advanced to a stage where it is possible to conduct a nearly complete survey of bacterial phagosome compositions and to perform a systematic interrogation of bacteria-mediated immunomodulation pathways.

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